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NOVEL MANNANASES

The present invention relates to microbial mannanases, more specifically to microbial enzymes exhibiting mannanase activity as their major enzymatic activity in the neutral and alkaline pH ranges; to a method of producing such enzymes; and to methods for using such enzymes in the paper and pulp, textile, cleaning and cellulose fiber processing industries.

BACKGROUND OF THE INVENTION

Mannan containing polysaccharides are a major component of the hemicellulose fraction in woods and endosperm in many leguminous seeds and in some mature seeds of non-leguminous plants. Essentially unsubstituted linear beta-1,4-mannan is found in some non-leguminous plants. Unsubstituted beta-1,4-mannan which is present e.g. in ivory nuts resembles cellulose in the conformation of the individual polysaccharide chains, and is water-insoluble. In leguminous seeds, water-soluble galactomannan is the main storage carbohydrate comprising up to 20% of the total dry weight. Galactomannans have a linear beta-1,4-mannan backbone substituted with single alpha-1,6-galactose, optionally substituted with acetyl groups. Mannans are also found in several monocotyledonous plants and are the most abundant polysaccharides in the cell wall material in palm kernel meal. Glucomannans are linear polysaccharides with a backbone of beta-1,4-linked mannose and glucose alternating in a more or less regular manner, the backbone optionally being substituted with galactose and/or acetyl groups. Mannans, galactomannans, glucomannans and galactoglucomannans (i.e. glucomannan backbones with branched galactose) contribute to more than 50% of the softwood hemicellulose. Moreover, the cellulose of many red algae contains a significant amount of

mannose.

Mannanases have been identified in several *Bacillus* organisms. For example, Talbot et al., Appl. Environ. Microbiol., Vol.56, No. 11, pp. 3505-3510 (1990) describes a beta-mannanase derived from *Bacillus stearothermophilus* in dimer form having molecular weight of 162 kDa and an optimum pH of 5.5-7.5. Mendoza et al., World J. Microbiol. Biotech., Vol. 10, No. 5, pp. 551-555 (1994) describes a beta-mannanase derived from *Bacillus subtilis* having a molecular weight of 38 kDa, an optimum activity at pH 5.0 and 55°C and a pI of 4.8. JP-0304706 discloses a beta-mannanase derived from *Bacillus sp.*, having a molecular weight of 37±3 kDa measured by gel filtration, an optimum pH of 8-10 and a pI of 5.3-5.4. JP-63056289 describes the production of an alkaline, thermostable beta-mannanase which hydrolyses beta-1,4-D-mannopyranoside bonds of e.g. mannans and produces manno-oligosaccharides. JP-63036774 relates to the *Bacillus* microorganism FERM P-8856 which produces beta-mannanse and beta-mannosidase at an alkaline pH. JP-08051975 discloses alkaline beta-mannanases from alkalophilic *Bacillus sp.* AM-001. A purified mannanase from *Bacillus amyloliquefaciens* useful in the bleaching of pulp and paper and a method of preparation thereof is disclosed in WO 97/11164. WO 91/18974 describes a hemicellulase such as a glucanase, xylanase or mannanase active at an extreme pH and temperature. WO 94/25576 discloses an enzyme from *Aspergillus aculeatus*, CBS 101.43, exhibiting mannanase activity which may be useful for degradation or modification of plant or algae cell wall material. WO 93/24622 discloses a mannanase isolated from *Trichoderma reseei* useful for bleaching lignocellulosic pulps.

WO 95/35362 discloses cleaning compositions containing plant cell wall degrading enzymes having pectinase and/or hemicellulase and optionally cellulase activity for the removal

of stains of vegetable origin and further discloses an alkaline mannanase from the strain C11SB.G17.

It is an object of the present invention to provide a novel and efficient enzyme exhibiting mannanase activity also in the alkaline pH range, e.g. when applied in cleaning compositions or different industrial processes.

SUMMARY OF THE INVENTION

The inventors have now found a novel enzyme having substantial mannanase activity, i.e. an enzyme exhibiting mannanase activity which may be obtained from a bacterial strain of the genus *Bacillus*, more specifically of the strain *Bacillus agaradherens*, and have succeeded in identifying a DNA sequence encoding such enzyme. The DNA sequence and the deduced amino acid sequence are listed in the sequence listing as SEQ ID No. 1 and 2, respectively. It is believed that the novel enzyme will be classified according to the Enzyme Nomenclature in the Enzyme Class EC 3.2.1.78.

In a first aspect, the present invention relates to a mannanase which is i) a polypeptide produced by *Bacillus agaradherens*, NCIMB 40482, or ii) a polypeptide comprising an amino acid sequence as shown in positions 32-343 of SEQ ID NO:2, or iii) an analogue of the polypeptide defined in i) or ii) which is at least 70% homologous with said polypeptide, is derived from said polypeptide by substitution, deletion or addition of one or several amino acids, or is immunologically reactive with a polyclonal antibody raised against said polypeptide in purified form.

Within one aspect, the present invention provides an isolated polynucleotide molecule selected from the group consisting of (a) polynucleotide molecules encoding a polypeptide having mannanase activity and comprising a sequence of nucleotides as

shown in SEQ ID NO: 1 from nucleotide 97 to nucleotide 1029; (b) species homologs of (a); (c) polynucleotide molecules that encode a polypeptide having mannanase activity that is at least 70% identical to the amino acid sequence of SEQ ID NO: 2 from amino acid residue 32 to amino acid residue 343; (d) molecules complementary to (a), (b) or (c); and (e) degenerate nucleotide sequences of (a), (b), (c) or (d).

The plasmid pSJ1678 comprising the polynucleotide molecule (the DNA sequence) encoding a mannanase of the present invention has been transformed into a strain of the *Escherichia coli* which was deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany, on 18 May 1998 under the deposition number DSM 12180.

Within another aspect of the invention there is provided an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment selected from the group consisting of (a) polynucleotide molecules encoding a polypeptide having mannanase activity and comprising a sequence of nucleotides as shown in SEQ ID NO: 1 from nucleotide 97 to nucleotide 1029; (b) species homologs of (a); (c) polynucleotide molecules that encode a polypeptide having mannanase activity that is at least 70% identical to the amino acid sequence of SEQ ID NO: 2 from amino acid residue 32 to amino acid residue 343; and (d) degenerate nucleotide sequences of (a), (b), or (c); and a transcription terminator.

Within yet another aspect of the present invention there is provided a cultured cell into which has been introduced an expression vector as disclosed above, wherein said cell expresses the polypeptide encoded by the DNA segment.

A further aspect of the present invention provides an isolated polypeptide having mannanase activity selected from the group consisting of (a) polypeptide molecules comprising a sequence of amino acid residues as shown in SEQ ID NO:2 from amino acid residue 32 to amino acid residue 343; (b) species homologs of (a).

Within another aspect of the present invention there is provided a composition comprising a purified polypeptide according to the invention in combination with other polypeptides.

Within another aspect of the present invention there are provided methods for producing a polypeptide according to the invention comprising culturing a cell into which has been introduced an expression vector as disclosed above, whereby said cell expresses a polypeptide encoded by the DNA segment and recovering the polypeptide.

The novel enzyme of the present invention is useful for the treatment of cellulosic material, especially cellulose-containing fiber, yarn, woven or non-woven fabric, treatment of mechanical paper-making pulps, kraft pulps or recycled waste paper, and for retting of fibres. The treatment can be carried out during the processing of cellulosic material into a material ready for manufacture of paper or of garment or fabric, the latter e.g. in the desizing or scouring step; or during industrial or household laundering of such fabric or garment.

Accordingly, in further aspects the present invention relates to a cleaning or detergent composition comprising an enzyme having substantial mannanase activity; and to use of the enzyme of the invention for the treatment, eg cleaning, of cellulose-containing fibers, yarn, woven or non-woven fabric, as well as synthetic or partly synthetic fabric.

The enzyme of the invention is very effective for use in an enzymatic scouring process and/or desizing (removal of mannan

size) in the preparation of cellulosic material e.g. for proper response in subsequent dyeing operations. The enzyme is also useful for removal of mannan containing print paste. Further, detergent compositions comprising the novel enzyme are capable of removing or bleaching certain soils or stains present on laundry, especially soils and spots resulting from mannan containing food, plants, and the like. Further, treatment with cleaning or detergent compositions comprising the novel enzyme can prevent binding of certain soils to the cellulosic material.

Accordingly, the present invention also relates to cleaning compositions, including laundry, dishwashing, hard surface cleaner, personal cleansing and oral/dental compositions, comprising a mannanase and a bioscouring enzyme selected from cellulases, amylases, pectin degrading enzymes and/or xyloglucanases, such compositions providing superior cleaning performance, i.e. superior stain removal, dingy cleaning and whiteness maintenance.

DEFINITIONS

Prior to discussing this invention in further detail, the following terms will first be defined.

The term "ortholog" (or "species homolog") denotes a polypeptide or protein obtained from one species that has homology to an analogous polypeptide or protein from a different species.

The term "paralog" denotes a polypeptide or protein obtained from a given species that has homology to a distinct polypeptide or protein from that same species.

The term "expression vector" denotes a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may include

promoter and terminator sequences, and may optionally include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both. The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which the vector is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The term "recombinant expressed" or "recombinantly expressed" used herein in connection with expression of a polypeptide or protein is defined according to the standard definition in the art. Recombinantly expression of a protein is generally performed by using an expression vector as described immediately above.

The term "isolated", when applied to a polynucleotide molecule, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated

regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985). The term "an isolated polynucleotide" may alternatively be termed "a cloned polynucleotide".

When applied to a protein/polypeptide, the term "isolated" indicates that the protein is found in a condition other than its native environment. In a preferred form, the isolated protein is substantially free of other proteins, particularly other homologous proteins (*i.e.* "homologous impurities" (see below)). It is preferred to provide the protein in a greater than 40% pure form, more preferably greater than 60% pure form.

Even more preferably it is preferred to provide the protein in a highly purified form, i.e., greater than 80% pure, more preferably greater than 95% pure, and even more preferably greater than 99% pure, as determined by SDS-PAGE.

The term "isolated protein/polypeptide may alternatively be termed "purified protein/polypeptide".

The term "homologous impurities" means any impurity (e.g. another polypeptide than the polypeptide of the invention) which originate from the homologous cell where the polypeptide of the invention is originally obtained from.

The term "obtained from" as used herein in connection with a specific microbial source, means that the polynucleotide and/or polypeptide produced by the specific source, or by a cell in which a gene from the source have been inserted.

The term "operably linked", when referring to DNA segments, denotes that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in the promoter and proceeds through the coding segment to the terminator

The term "polynucleotide" denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases

read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules.

The term "complements of polynucleotide molecules" denotes polynucleotide molecules having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "promoter" denotes a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger peptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "mannanase" or "galactomannanase" denotes a mannanase enzyme defined according to the art as officially being named mannan endo-1,4-beta-mannosidase and having the alternative names beta-mannanase and endo-1,4-mannanase and catalysing the reaction: random hydrolyses of 1,4-beta-D-mannosidic linkages in mannans, galactomannans, glucomannans, and galactoglucomannans.

DETAILED DESCRIPTION OF THE INVENTION

HOW TO USE A SEQUENCE OF THE INVENTION TO GET OTHER RELATED SEQUENCES: The disclosed sequence information herein relating to a polynucleotide sequence encoding a mannanase of the invention can be used as a tool to identify other homologous mannanases. For instance, polymerase chain reaction (PCR) can be used to amplify sequences encoding other homologous mannanases from a variety of microbial sources, in particular of different *Bacillus* species.

ASSAY FOR ACTIVITY TEST

A polypeptide of the invention having mannanase activity may be tested for mannanase activity according to standard test procedures known in the art, such as by applying a solution to be tested to 4 mm diameter holes punched out in agar plates containing 0.2% AZCL galactomannan (carob), i.e. substrate for the assay of endo-1,4-beta-D-mannanase available as CatNo.I-AZGMA from the company Megazyme (Megazyme's Internet address: http://www.megazyme.com/Purchase/index.html).

POLYNUCLEOTIDES:

Within preferred embodiments of the invention an isolated polynucleotide of the invention will hybridize to similar sized regions of SEQ ID No. 1, or a sequence complementary thereto, under at least medium stringency conditions.

In particular polynucleotides of the invention will hybridize to a denatured double-stranded DNA probe comprising either the full sequence shown in positions 97-1029 of SEQ ID NO:1 or any probe comprising a subsequence of SEQ ID NO:1 having a length of at least about 100 base pairs under at least medium stringency conditions, but preferably at high stringency

conditions as described in detail below. Suitable experimental conditions for determining hybridization at medium, or high stringency between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al. 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) Anal. Biochem. 132:6-13), 32P-dCTP-labeled (specific activity higher than 1 x 109 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at least 60°C (medium stringency), still more preferably at least 65°C (medium/high stringency), even more preferably at least 70°C (high stringency), and even more preferably at least 75°C (very high stringency).

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. DNA and RNA encoding genes of interest can be cloned in Gene Banks or DNA libraries by means of methods known in the art.

Polynucleotides encoding polypeptides having mannanase activity of the invention are then identified and isolated by, for example, hybridization or PCR.

The present invention further provides counterpart polypeptides and polynucleotides from different bacterial strains (orthologs or paralogs). Of particular interest are mannanase polypeptides from gram-positive alkalophilic strains,

including species of *Bacillus*.

Species homologues of a polypeptide with mannanase activity of the invention can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a DNA sequence of the present invention can be cloned using chromosomal DNA obtained from a cell type that expresses the protein. Suitable sources of DNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from chromosomal DNA of a positive cell line. A DNA sequence of the invention encoding an polypeptide having mannanase activity can then be isolated by a variety of methods, such as by probing with probes designed from the sequences disclosed in the present specification and claims or with one or more sets of degenerate probes based on the disclosed sequences. A DNA sequence of the invention can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent 4,683,202), using primers designed from the sequences disclosed herein. Within an additional method, the DNA library can be used to transform or transfect host cells, and expression of the DNA of interest can be detected with an antibody (monoclonal or polyclonal) raised against the mannanase cloned from *B.agaradherens,* NCIMB 40482, expressed and purified as described in Materials and Methods and Example 1, or by an activity test relating to a polypeptide having mannanase activity.

The mannanase encoding part of the DNA sequence cloned into plasmid pSJ1678 present in *Escherichia coli* DSM 12180 and/or an analogue DNA sequence of the invention may be cloned from a strain of the bacterial species *Bacillus agaradherens,* preferably the strain NCIMB 40482, producing the enzyme with mannan degrading activity, or another or related organism as described herein.

Alternatively, the analogous sequence may be constructed on the basis of the DNA sequence obtainable from the plasmid present in *Escherichia coli* DSM 12180 (which is believed to be identical to the attached SEQ ID NO:1), *e.g* be a sub-sequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the mannanase encoded by the DNA sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence (*i.e.* a variant of the mannan degrading enzyme of the invention).

POLYPEPTIDES:

The sequence of amino acids nos. 32-343 of SEQ ID NO: 2 is a mature mannanase sequence.

The present invention also provides mannanase polypeptides that are substantially homologous to the polypeptide of SEQ ID NO:2 and species homologs (paralogs or orthologs) thereof. The term "substantially homologous" is used herein to denote polypeptides having 70%, preferably at least 80%, more preferably at least 85%, and even more preferably at least 90%, sequence identity to the sequence shown in amino acids nos. 32-343 of SEQ ID NO:2 or their orthologs or paralogs. Such polypeptides will more preferably be at least 95% identical, and most preferably 98% or more identical to the sequence shown in amino acids nos. 32-343 of SEQ ID NO:2 or its orthologs or paralogs. Percent sequence identity is determined by conventional methods, by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) as disclosed in Needleman, S.B. and Wunsch, C.D.,

(1970), Journal of Molecular Biology, 48, 443-453, which is hereby incorporated by reference in its entirety. GAP is used with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Sequence identity of polynucleotide molecules is determined by similar methods using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

The enzyme preparation of the invention is preferably derived from a microorganism, preferably from a bacterium, an archea or a fungus, especially from a bacterium such as a bacterium belonging to *Bacillus*, preferably to an alkalophilic *Bacillus* strain which may be selected from the group consisting of the species *Bacillus agaradherens* and highly related *Bacillus* species in which all species preferably are at least 95%, even more preferably at least 98%, homologous to *Bacillus agaradherens* based on aligned 16S rDNA sequences.

Substantially homologous proteins and polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 2) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification (an affinity tag), such as a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991. See, in general Ford et al., Protein Expression and Purification 2: 95-107, 1991, which is incorporated herein by reference. DNAs encoding affinity tags

are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ; New England Biolabs, Beverly, MA).

However, even though the changes described above preferably are of a minor nature, such changes may also be of a larger nature such as fusion of larger polypeptides of up to 300 amino acids or more both as amino- or carboxyl-terminal extensions to a Mannanase polypeptide of the invention.

Table 1

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-*N*-methyl lysine, 2-aminoisobutyric acid, isovaline and a-methyl serine) may be

substituted for amino acid residues of a polypeptide according to the invention. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for amino acid residues. "Unnatural amino acids" have been modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, or preferably, are commercially available, and include pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

Essential amino acids in the mannanase polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-1085, 1989). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (*i.e* mannanase activity) to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., J. Biol. Chem. 271:4699-4708, 1996. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-312, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992.

The identities of essential amino acids can also be inferred from analysis of homologies with polypeptides which are related to a polypeptide according to the invention.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis, recombination and/or shuffling followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-57, 1988), Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-2156, 1989), WO95/17413, or WO 95/22625. Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, or recombination/shuffling of different mutations (WO95/17413, WO95/22625), followed by selecting for functional a polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-10837, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

Mutagenesis/shuffling methods as disclosed above can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

Using the methods discussed above, one of ordinary skill in the art can identify and/or prepare a variety of polypeptides that are substantially homologous to residues 32 to 343 of SEQ ID NO: 2 and retain the mannanase activity of the wild-type protein.

The mannanase enzyme of the invention may, in addition to the enzyme core comprising the catalytically domain, also com-

prise a cellulose binding domain (CBD), the cellulose binding domain and enzyme core (the catalytically active domain) of the enzyme being operably linked. The cellulose binding domain (CBD) may exist as an integral part of the encoded enzyme, or a CBD from another origin may be introduced into the mannan degrading enzyme thus creating an enzyme hybrid. In this context, the term "cellulose-binding domain" is intended to be understood as defined by Peter Tomme et al. "Cellulose-Binding Domains: Classification and Properties" in "Enzymatic Degradation of Insoluble Carbohydrates", John N. Saddler and Michael H. Penner (Eds.), ACS Symposium Series, No. 618, 1996. This definition classifies more than 120 cellulose-binding domains into 10 families (I-X), and demonstrates that CBDs are found in various enzymes such as cellulases, xylanases, mannanases, arabinofuranosidases, acetyl esterases and chitinases. CBDs have also been found in algae, e.g. the red alga *Porphyra purpurea* as a non-hydrolytic polysaccharide-binding protein, see Tomme et al., *op.cit*. However, most of the CBDs are from cellulases and xylanases, CBDs are found at the N and C termini of proteins or are internal. Enzyme hybrids are known in the art, see e.g. WO 90/00609 and WO 95/16782, and may be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the cellulose-binding domain ligated, with or without a linker, to a DNA sequence encoding the mannan degrading enzyme and growing the host cell to express the fused gene. Enzyme hybrids may be described by the following formula:

CBD - MR - X

wherein CBD is the N-terminal or the C-terminal region of an amino acid sequence corresponding to at least the cellulose-binding domain; MR is the middle region (the linker), and may be a bond, or a short linking group preferably of from about 2 to

about 100 carbon atoms, more preferably of from 2 to 40 carbon atoms; or is preferably from about 2 to to about 100 amino acids, more preferably of from 2 to 40 amino acids; and X is an N-terminal or C-terminal region of the mannanase of the invention.

Preferably, the mannanase enzyme of the present invention has its maximum catalytic activity at a pH of at least 8, more preferably of at least 8.5, more preferably of at least 9, more preferably of at least 9.5, more preferably of at least 10, even more preferably of at least 10.5, especially of at least 11; and preferably the maximum activity of the enzyme is obtained at a temperature of at least 50°C, more preferably of at least 55°C.

PROTEIN PRODUCTION:

The proteins and polypeptides of the present invention, including full-length proteins, fragments thereof and fusion proteins, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Bacterial cells, particularly cultured cells of gram-positive organisms, are preferred. Gram-positive cells from the genus of Bacillus are especially preferred, such as from the group consisting of *Bacillus subtilis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus thuringiensis, Bacillus licheniformis,* and *Bacillus agaradherens,* in particular *Bacillus agaradherens.*

Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd

ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987; and "Bacillus subtilis and Other Gram-Positive Bacteria", Sonensheim et al., 1993, American Society for Microbiology, Washington D.C., which are incorporated herein by reference.

In general, a DNA sequence encoding a mannanase of the present invention is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of the polypeptide, or may be derived from another secreted protein or synthesized *de novo*. Numerous suitable secretory signal sequences are known in the art and reference is made to "Bacillus subtilis and Other Gram-Positive Bacteria", Sonensheim et al., 1993, American Society for Microbiology, Washington D.C.; and Cutting, S. M.(eds.) "Molecular Biological Methods for Bacillus", John Wiley and Sons, 1990, for further description of suitable secretory signal sequences especially for secretion in

a Bacillus host cell. The secretory signal sequence is joined to the DNA sequence in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

PROTEIN ISOLATION:

When the expressed recombinant polypeptide is secreted the polypeptide may be purified from the growth media. Preferably the expression host cells are removed from the media before purification of the polypeptide (*e.g.* by centrifugation).

When the expressed recombinant polypeptide is not secreted from the host cell, the host cell are preferably disrupted and the polypeptide released into an aqueous "extract" which is the first stage of such purification techniques. Preferably the expression host cells are collected from the media before the cell disruption (*e.g.* by centrifugation).

The cell disruption may be performed by conventional techniques such as by lysozyme digestion or by forcing the cells through high pressure. See (Robert K. Scobes, Protein Purification, Second edition, Springer-Verlag) for further description of such cell disruption techniques.

Whether or not the expressed recombinant polypeptides (or chimeric polypeptides) is secreted or not it can be purified using fractionation and/or conventional purification methods and media.

Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable anion exchange media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred, with DEAE Fast-Flow Sepharose (Pharmacia, Piscataway, NJ) being particularly preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and

carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers.

Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988.

Polypeptides of the invention or fragments thereof may also be prepared through chemical synthesis. Polypeptides of the invention may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue.

Based on the sequence information disclosed herein a full length DNA sequence encoding a mannanase of the invention and comprising the DNA sequence shown in SEQ ID No 1, at least the DNA sequence from position 97 to position 1029, may be cloned.

Cloning is performed by standard procedures known in the art such as by,

- preparing a genomic library from a *Bacillus* strain, especially the strain *B. agaradherens*, NCIMB 40482;
- plating such a library on suitable substrate plates;
- identifying a clone comprising a polynucleotide sequence of the invention by standard hybridization techniques using a probe based on SEQ ID No 1; or by
- identifying a clone from said *Bacillus agaradherens* NCIMB 40482 genomic library by an Inverse PCR strategy using primers based on sequence information from SEQ ID No 1. Reference is made to M.J. MCPherson et al. ("PCR A practical approach" Information Press Ltd, Oxford England) for further details relating to Inverse PCR.

Based on the sequence information disclosed herein (SEQ ID No 1, SEQ ID No 2) is it routine work for a person skilled in the art to isolate homologous polynucleotide sequences encoding homologous mannanase of the invention by a similar strategy using genomic libraries from related microbial organisms, in particular from genomic libraries from other strains of the genus *Bacillus* such as alkalophilic species of *Bacillus*.

Alternatively, the DNA encoding the mannan or galactomannan-degrading enzyme of the invention may, in accordance with well-known procedures, conveniently be cloned from a suitable source, such as any of the above mentioned organisms, by use of synthetic oligonucleotide probes prepared on the basis of the DNA sequence obtainable from the plasmid present in *Escherichia coli* DSM 12180.

Accordingly, the polynucleotide molecule of the invention may be isolated from *Escherichia coli*, DSM 12180, in which the plasmid obtained by cloning such as described above is deposited. Also, the present invention relates to an isolated substantially pure biological culture of the strain *Escherichia coli*, DSM 12180.

In the present context, the term "enzyme preparation" is intended to mean either a conventional enzymatic fermentation product, possibly isolated and purified, from a single species of a microorganism, such preparation usually comprising a number of different enzymatic activities; or a mixture of monocomponent enzymes, preferably enzymes derived from bacterial or fungal species by using conventional recombinant techniques, which enzymes have been fermented and possibly isolated and purified separately and which may originate from different species, preferably fungal or bacterial species; or the fermentation product of a microorganism which acts as a host cell for expression of a recombinant mannanase, but which microorganism

simultaneously produces other enzymes, e.g. pectin degrading enzymes, proteases, or cellulases, being naturally occurring fermentation products of the microorganism, i.e. the enzyme complex conventionally produced by the corresponding naturally occurring microorganism.

The mannanase preparation of the invention may further comprise one or more enzymes selected from the group consisting of proteases, cellulases (endo-β-1,4-glucanases), β-glucanases (endo-β-1,3(4)-glucanases), lipases, cutinases, peroxidases, laccases, amylases, glucoamylases, pectinases, reductases, oxidases, phenoloxidases, ligninases, pullulanases, hemicellulases, pectate lyases, xyloglucanases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, polygalacturonases, rhamnogalacturonases, pectin lyases, pectin methylesterases, cellobiohydrolases, transglutaminases; or mixtures thereof. In a preferred embodiment, one or more or all enzymes in the preparation is produced by using recombinant techniques, i.e. the enzyme(s) is/are mono-component enzyme(s) which is/are mixed with the other enzyme(s) to form an enzyme preparation with the desired enzyme blend.

In another aspect, the present invention also relates to a method of producing the enzyme preparation of the invention, the method comprising culturing a microorganism, eg a wild-type strain, capable of producing the mannanase under conditions permitting the production of the enzyme, and recovering the enzyme from the culture. Culturing may be carried out using conventional fermentation techniques, e.g. culturing in shake flasks or fermentors with agitation to ensure sufficient aeration on a growth medium inducing production of the mannanase enzyme. The growth medium may contain a conventional N-source such as peptone, yeast extract or casamino acids, a reduced amount of a conventional C-source such as dextrose or sucrose,

and an inducer such as guar gum or locust bean gum. The recovery may be carried out using conventional techniques, e.g. separation of bio-mass and supernatant by centrifugation or filtration, recovery of the supernatant or disruption of cells if the enzyme of interest is intracellular, perhaps followed by further purification as described in EP 0 406 314 or by crystallization as described in WO 97/15660.

IMMUNOLOGICAL CROSS-REACTIVITY:

Polyclonal antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified mannanase enzyme. More specifically, antiserum against the mannanase of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen et al. in: A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically p. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2$ SO_4), followed by dialysis and ion exchange chromatography, *e.g.* on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

Examples of useful bacteria producing the enzyme or the enzyme preparation of the invention are Gram positive bacteria, preferably from the *Bacillus/Lactobacillus* subdivision,

preferably a strain from the genus *Bacillus*, more preferably a strain of *Bacillus agaradherens*, esepcially the strain *Bacillus agaradherens*, NCIMB 40482.

In yet another aspect, the present invention relates to an isolated mannanase having the properties described above and which is free from homologous impurities, and is produced using conventional recombinant techniques.

Use in the detergent industry

In further aspects, the present invention relates to a detergent composition comprising the mannanase or mannanase preparation of the invention, to a process for machine treatment of fabrics comprising treating fabric during a washing cycle of a machine washing process with a washing solution containing the mannanase or mannanase preparation of the invention, and to cleaning compositions, including laundry, dishwashing, hard surface cleaner, personal cleansing and oral/dental compositions, comprising a mannanase and a bioscouring enzyme selected from cellulases, amylases, pectin degrading enzymes and/or xyloglucanases and providing superior cleaning performance, i.e. superior stain removal, dingy cleaning and whiteness maintenance.

Without being bound to this theory, it is believed that the mannanase of the present invention is capable of effectively degrading or hydrolysing any soiling or spots containing galatomannans and, accordingly, of cleaning laundry comprising such soilings or spots.

The cleaning compositions of the invention must contain at least one additional detergent component. The precise nature of these additional components, and levels of incorporation thereof will depend on the physical form of the composition, and the nature of the cleaning operation for which it is to be used.

The cleaning compositions of the present invention preferably further comprise a detergent ingredient selected from a selected surfactant, another enzyme, a builder and/or a bleach system.

The cleaning compositions according to the invention can be liquid, paste, gels, bars, tablets, spray, foam, powder or granular. Granular compositions can also be in "compact" form and the liquid compositions can also be in a "concentrated" form.

The compositions of the invention may for example, be formulated as hand and machine dishwashing compositions, hand and machine laundry detergent compositions including laundry additive compositions and compositions suitable for use in the soaking and/or pretreatment of stained fabrics, rinse added fabric softener compositions, and compositions for use in general household hard surface cleaning operations. Compositions containing such carbohydrases can also be formulated as sanitization products, contact lens cleansers and health and beauty care products such as oral/dental care and personal cleaning compositions.

When formulated as compositions for use in manual dishwashing methods the compositions of the invention preferably contain a surfactant and preferably other detergent compounds selected from organic polymeric compounds, suds enhancing agents, group II metal ions, solvents, hydrotropes and additional enzymes.

When formulated as compositions suitable for use in a laundry machine washing method, the compositions of the invention preferably contain both a surfactant and a builder compound and additionally one or more detergent components preferably selected from organic polymeric compounds, bleaching agents, additional enzymes, suds suppressors, dispersants, lime-soap dispersants, soil suspension and anti-redeposition agents and

corrosion inhibitors. Laundry compositions can also contain softening agents, as additional detergent components. Such compositions containing carbohydrase can provide fabric cleaning, stain removal, whiteness maintenance, softening, colour appearance, dye transfer inhibition and sanitization when formulated as laundry detergent compositions.

The compositions of the invention can also be used as detergent additive products in solid or liquid form. Such additive products are intended to supplement or boost the performance of conventional detergent compositions and can be added at any stage of the cleaning process.

If needed the density of the laundry detergent compositions herein ranges from 400 to 1200 g/litre, preferably 500 to 950 g/litre of composition measured at 20°C.

The "compact" form of the compositions herein is best reflected by density and, in terms of composition, by the amount of inorganic filler salt; inorganic filler salts are conventional ingredients of detergent compositions in powder form; in conventional detergent compositions, the filler salts are present in substantial amounts, typically 17-35% by weight of the total composition. In the compact compositions, the filler salt is present in amounts not exceeding 15% of the total composition, preferably not exceeding 10%, most preferably not exceeding 5% by weight of the composition. The inorganic filler salts, such as meant in the present compositions are selected from the alkali and alkaline-earth-metal salts of sulphates and chlorides. A preferred filler salt is sodium sulphate.

Liquid detergent compositions according to the present invention can also be in a "concentrated form", in such case, the liquid detergent compositions according the present invention will contain a lower amount of water, compared to conventional liquid detergents. Typically the water content of the

concentrated liquid detergent is preferably less than 40%, more preferably less than 30%, most preferably less than 20% by weight of the detergent composition.

Suitable specific detergent compounds for use herein are selected from the group consisting of the specific compounds as described in WO 97/01629 which is hereby incorporated by reference in its entirety.

Mannanase is incorporated into the cleaning compositions in accordance with the invention preferably at a level of from 0.0001% to 2%, more preferably from 0.0005% to 0.5%, most preferred from 0.001% to 0.1% pure enzyme by weight of the composition.

The cleaning compositions of the present invention further comprise as an essential element a bioscouring carbohydrase selected from cellulases, amylases, pectin degrading enzymes and/or xyloglucanases. Preferably, the cleaning compositions of the present invention will comprise a mannanase, an amylase and abother bioscouring enzyme selected from cellulases, pectin degrading enzymes and/or xyloglucanases.

The cellulases usable in the present invention include both bacterial or fungal cellulases. Preferably, they will have a pH optimum of between 5 and 12 and a specific activity above 50 CEVU/mg (Cellulose Viscosity Unit). Suitable cellulases are disclosed in U.S. Patent 4,435,307, J61078384 and WO96/02653 which discloses fungal cellulase produced from *Humicola insolens, Trichoderma, Thielavia* and *Sporotrichum*, respectively. EP 739 982 describes cellulases isolated from novel *Bacillus* species. Suitable cellulases are also disclosed in GB-A-2075028; GB-A-2095275; DE-OS-22 47 832 and WO95/26398.

Examples of such cellulases are cellulases produced by a strain of *Humicola insolens* (*Humicola grisea var. thermoidea*), particularly the strain *Humicola insolens*, DSM 1800. Other

suitable cellulases are cellulases originated from *Humicola insolens* having a molecular weight of about 50kD, an isoelectric point of 5.5 and containing 415 amino acids; and a ~43kD endo-beta-1,4-glucanase derived from *Humicola insolens*, DSM 1800; a preferred cellulase has the amino acid sequence disclosed in PCT Patent Application No. WO 91/17243. Also suitable cellulases are the EGIII cellulases from *Trichoderma longibrachiatum* described in WO94/21801. Especially suitable cellulases are the cellulases having color care benefits. Examples of such cellulases are the cellulases described in WO96/29397, EP-A-0495257, WO 91/17243, WO91/17244 and WO91/21801. Other suitable cellulases for fabric care and/or cleaning properties are described in WO96/34092, WO96/17994 and WO95/24471.

Said cellulases are normally incorporated in the detergent composition at levels from 0.0001% to 2% of pure enzyme by weight of the detergent composition.

Preferred cellulases for the purpose of the present invention are alkaline cellulases, i.e. enzyme having at least 25%, more preferably at least 40% of their maximum activity at a pH ranging from 7 to 12. More preferred cellulases are enzymes having their maximum activity at a pH ranging from 7 to 12. A preferred alkaline cellulase is the cellulase sold under the tradename Carezyme® by Novo Nordisk A/S.

Amylases (α and/or ß) can be included for removal of carbohydrate-based stains. WO94/02597, Novo Nordisk A/S published February 03, 1994, describes cleaning compositions which incorporate mutant amylases. See also WO95/10603, Novo Nordisk A/S, published April 20, 1995. Other amylases known for use in cleaning compositions include both α- and β-amylases. α-Amylases are known in the art and include those disclosed in US Pat. no. 5,003,257; EP 252,666; WO/91/00353; FR 2,676,456; EP 285,123; EP 525,610; EP 368,341; and British Patent specification no.

1,296,839 (Novo). Other suitable amylases are stability-enhanced amylases described in WO94/18314, published August 18, 1994 and WO96/05295, Genencor, published February 22, 1996 and amylase variants having additional modification in the immediate parent available from Novo Nordisk A/S, disclosed in WO 95/10603, published April 95. Also suitable are amylases described in EP 277 216, WO95/26397 and WO96/23873 (all by Novo Nordisk).

Examples of commercial α-amylases products are Purafect Ox Am® from Genencor and Termamyl®, Ban® ,Fungamyl® and Duramyl®, all available from Novo Nordisk A/S Denmark. WO95/26397 describes other suitable amylases : α-amylases characterised by having a specific activity at least 25% higher than the specific activity of Termamyl® at a temperature range of 25°C to 55°C and at a pH value in the range of 8 to 10, measured by the Phadebas ® α-amylase activity assay. Suitable are variants of the above enzymes, described in WO96/23873 (Novo Nordisk). Other amylolytic enzymes with improved properties with respect to the activity level and the combination of thermostability and a higher activity level are described in WO95/35382.

Preferred amylases for the purpose of the present invention are the amylases sold under the tradename Termamyl, Duramyl and Maxamyl and or the α-amylase variant demonstrating increased thermostability disclosed as SEQ ID No. 2 in WO96/23873.

Preferred amylases for specific applications are alkaline amylases, ie enzymes having an enzymatic activity of at least 10%, preferably at least 25%, more preferably at least 40% of their maximum activity at a pH ranging from 7 to 12. More preferred amylases are enzymes having their maximum activity at a pH ranging from 7 to 12.

The amylolytic enzymes are incorporated in the detergent compositions of the present invention a level of from 0.0001% to

2%, preferably from 0.00018% to 0.06%, more preferably from 0.00024% to 0.048% pure enzyme by weight of the composition.

The term "pectin degrading enzyme" is intended to encompass polygalacturonase (EC 3.2.1.15) exo-polygalacturonase (EC 3.2.1.67), exo-poly-alpha-galacturonidase (EC 3.2.1.82), pectin lyase (EC 4.2.2.10), pectin esterase (EC 3.2.1.11), pectate lyase (EC 4.2.2.2), exo-polygalacturonate lyase (EC 4.2.2.9)and hemicellulases such as endo-1,3-β-xylosidase (EC 3.2.1.32), xylan-1,4-β-xylosidase (EC 3.2.1.37)and α-L-arabinofuranosidase (EC 3.2.1.55). The pectin degrading enzymes are natural mixtures of the above mentioned enzymatic activities. Pectin enzymes therefore include the pectin methylesterases which hydrolyse the pectin methyl ester linkages, polygalacturonases which cleave the glycosidic bonds between galacturonic acid molecules, and the pectin transeliminases or lyases which act on the pectic acids to bring about non-hydrolytic cleavage of α-1→4 glycosidic linkages to form unsaturated derivatives of galacturonic acid.

Pectin degrading enzymes are incorporated into the compositions in accordance with the invention preferably at a level of from 0.0001 % to 2 %, more preferably from 0.0005% to 0.5%, most preferred from 0.001 % to 0.1 % pure enzyme by weight of the total composition.

Preferred pectin degrading enzymes for specific applications are alkaline pectin degrading enzymes, ie enzymes having an enzymatic activity of at least 10%, preferably at least 25%, more preferably at least 40% of their maximum activity at a pH ranging from 7 to 12. More preferred pectin degrading enzymes are enzymes having their maximum activity at a pH ranging from 7 to 12. Alkaline pectin degrading enzymes are produced by alkalophilic microorganisms e.g. bacterial, fungal and yeast microorganisms such as *Bacillus* species. Preferred microorganisms are

Bacillus firmus, Bacillus circulans and *Bacillus subtilis* as described in JP 56131376 and JP 56068393. Alkaline pectin decomposing enzymes include galacturan-1,4-α-galacturonase (EC 3.2.1.67), poly-galacturonase activities (EC 3.2.1.15, pectin esterase (EC 3.1.1.11), pectate lyase (EC 4.2.2.2) and their iso enzymes and they can be produced by the *Erwinia* species. Preferred are *E. chrysanthemi, E. carotovora, E. amylovora, E. herbicola, E. dissolvens* as described in JP 59066588, JP 63042988 and in World J. Microbiol. Microbiotechnol. (8, 2, 115-120) 1992. Said alkaline pectin enzymes can also be produced by *Bacillus species* as disclosed in JP 73006557 and Agr. Biol. Chem. (1972), 36(2) 285-93.

The term xyloglucanase encompasses the family of enzymes described by Vincken and Voragen at Wageningen University [Vincken et al (1994) Plant Physiol., **104**, 99-107] and are able to degrade xyloglucans as described in Hayashi et al (1989) Plant. Physiol. Plant Mol. Biol., **40**, 139-168. Vincken et al demonstrated the removal of xyloglucan coating from cellulase of the isolated apple cell wall by a xyloglucanase purified from *Trichoderma viride* (endo-IV-glucanase). This enzyme enhances the enzymatic degradation of cell wall-embedded cellulose and work in synergy with pectic enzymes. Rapidase LIQ+ from Gist-Brocades contains an xyloglucanase activity.

This xyloglucanase is incorporated into the cleaning compositions in accordance with the invention preferably at a level of from 0.0001% to 2%, more preferably from0.0005% to 0.5%, most preferred from 0.001% to0.1 % pure enzyme by weight of the composition.

Preferred xyloglucanases for specific applications are alkaline xyloglucanases, ie enzymes having an enzymatic activity of at least 10%, preferably at least 25%, more preferably at least 40% of their maximum activity at a pH ranging from 7 to

12. More preferred xyloglucanases are enzymes having their maximum activity at a pH ranging from 7 to 12.

The above-mentioned enzymes may be of any suitable origin, such as vegetable, animal, bacterial, fungal and yeast origin. Origin can further be mesophilic or extremophilic (psychrophilic, psychrotrophic, thermophilic, barophilic, alkalophilic, acidophilic, halophilic, etc.). Purified or non-purified forms of these enzymes may be used. Nowadays, it is common practice to modify wild-type enzymes via protein / genetic engineering techniques in order to optimise their performance efficiency in the cleaning compositions of the invention. For example, the variants may be designed such that the compatibility of the enzyme to commonly encountered ingredients of such compositions is increased. Alternatively, the variant may be designed such that the optimal pH, bleach or chelant stability, catalytic activity and the like, of the enzyme variant is tailored to suit the particular cleaning application.

In particular, attention should be focused on amino acids sensitive to oxidation in the case of bleach stability and on surface charges for the surfactant compatibility. The isoelectric point of such enzymes may be modified by the substitution of some charged amino acids, e.g. an increase in isoelectric point may help to improve compatibility with anionic surfactants. The stability of the enzymes may be further enhanced by the creation of e.g. additional salt bridges and enforcing metal binding sites to increase chelant stability.

Use in the paper pulp industry

Further, it is contemplated that the mannanase of the present invention is useful in chlorine-free bleaching processes for paper pulp (chemical pulps, semichemical pulps, mechanical pulps or kraft pulps) in order to increase the brightness

thereof, thus decreasing or eliminating the need for hydrogen peroxide in the bleaching process.

Use in the textile and cellulosic fiber processing industries

The mannanase of the present invention can be used in combination with other carbohydrate degrading enzymes (for instance xyloglucanase, xylanase, various pectinases) for preparation of fibers or for cleaning of fibers in combination with detergents.

In the present context, the term "cellulosic material" is intended to mean fibers, sewn and unsewn fabrics, including knits, wovens, denims, yarns, and toweling, made from cotton, cotton blends or natural or manmade cellulosics (e.g. originating from xylan-containing cellulose fibers such as from wood pulp) or blends thereof. Examples of blends are blends of cotton or rayon/viscose with one or more companion material such as wool, synthetic fibers (e.g. polyamide fibers, acrylic fibers, polyester fibers, polyvinyl alcohol fibers, polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers, polyurea fibers, aramid fibers), and cellulose-containing fibers (e.g. rayon/viscose, ramie, hemp, flax/linen, jute, cellulose acetate fibers, lyocell).

The processing of cellulosic material for the textile industry, as for example cotton fiber, into a material ready for garment manufacture involves several steps: spinning of the fiber into a yarn; construction of woven or knit fabric from the yarn and subsequent preparation, dyeing and finishing operations. Woven goods are constructed by weaving a filling yarn between a series of warp yarns; the yarns could be two different types.

Desizing: polymeric size like e.g. mannan, starch, CMC or PVA is added before weaving in order to increase the warp speed; This material must be removed before further processing. The

enzyme of the invention is useful for removal of mannan containing size.

Degradation of thickeners

Galactomannans such as guar gum and locust bean gum are widely used as thickening agents e.g. in food and print paste for textile printing such as prints on T-shirts. The enzyme or enzyme preparation according to the invention can be used for reducing the viscosity of eg residual food in processing equipment and thereby facilitate cleaning after processing. Further, it is contemplated that the enzyme or enzyme preparation is useful for reducing viscosity of print paste, thereby facilitating wash out of surplus print paste after textile printins.

Degradation or modification of plant material

The enzyme or enzyme preparation according to the invention is preferably used as an agent for degradation or modification of mannan, galactomannan, glucomannan or galactoglucomannan containing material originating from plants. Examples of such material is guar gum and locust bean gum.

The mannanase of the invention may be used in modifying the physical-chemical properties of plant derived material such as the viscosity. For instance, the mannanase may be used to reduce the viscosity of feed or food which contain mannan and to promote processing of viscous mannan containing material.

Coffee extraction

The enzyme or enzyme preparation of the invention may also be used for hydrolysing galactomannans present in a liquid coffee extract, preferably in order to inhibit gel formation during freeze drying of the (instant) coffee. Preferably, the mannanase of the invention is immobilized in order to reduce enzyme

consumption and avoid contamination of the coffee. This use is further disclosed in EP-A-676 145.

Use in the fracturing of a subterranean formation (oil drilling)

Further, it is contemplated that the enzyme of the present invention is useful as an enzyme breaker as disclosed in US patent nos. 5,806,597, 5,562,160, 5,201,370 and 5,067,566 to BJ Services Company (Houston, TX, U.S.A.) all of which are hereby incorporated by reference.

Accordingly, the mannanase of the present invention is useful in a method of fracturing a subterranean formation in a well bore in which a gellable fracturing fluid is first formed by blending together an aqueous fluid, a hydratable polymer, a suitable cross-linking agent for cross-linking the hydratable polymer to form a polymer gel and an enzyme breaker, ie the enzyme of the invention. The cross-linked polymer gel is pumped into the well bore under sufficient pressure to fracture the surrounding formation. The enzyme breaker is allowed to degrade the cross-linked polymer with time to reduce the viscosity of the fluid so that the fluid can be pumped from the formation back to the well surface.

The enzyme breaker may be an ingredient of a fracturing fluid or a breaker-crosslinker-polymer complex which further comprises a hydratable polymer and a crosslinking agent. The fracturing fluid or complex may be a gel or may be gellable. The complex is useful in a method for using the complex in a fracturing fluid to fracture a subterranean formation that surrounds a well bore by pumping the fluid to a desired location within the well bore under sufficient pressure to fracture the surrounding subterranean formation. The complex may be maintained in a substantially non-reactive state by maintaining specific conditions of pH and temperature, until a time at which the

fluid is in place in the well bore and the desired fracture is completed. Once the fracture is completed, the specific conditions at which the complex is inactive are no longer maintained. When the conditions change sufficiently, the complex becomes active and the breaker begins to catalyze polymer degradation causing the fracturing fluid to become sufficiently fluid to be pumped from the subterranean formation to the well surface.

MATERIALS AND METHODS

Determination of catalytic activity (ManU) of mannanase

Colorimetric Assay

Substrate: 0.2% AZCL-Galactomannan (Megazyme, Australia) from carob in 0.1 M Glycin buffer, pH 10.0.

The assay is carried out in an Eppendorf Micro tube 1.5 ml on a thermomixer with stirring and temperature control of 40°C. Incubation of 0.750 ml substrate with 0.05 ml enzyme for 20 min, stop by centrifugation for 4 minutes at 15000 rpm. The colour of the supernatant is measured at 600 nm in a 1 cm cuvette.

One ManU (Mannanase units) gives 0.24 abs in 1 cm.

Strains

Bacillus agaradherens NCIMB 40482 comprises the mannanase enzyme encoding DNA sequence.

E. coli strain: Cells of E. coli SJ2 (Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B. R., Sjøholm, C. (1990) Cloning of aldB, which encodes alpha-acetolactate decarboxylase, an exoenzyme from Bacillus brevis. J. Bacteriol., 172, 4315-4321), were prepared for and transformed by electroporation using a Gene Pulser™ electroporator from BIO-RAD as described by the supplier.

B.subtilis PL2306. This strain is the B.subtilis DN1885 with disrupted apr and npr genes (Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B. R., Sjøholm, C. (1990) Cloning of aldB, which encodes alpha-acetolactate decarboxylase, an exoenzyme from Bacillus brevis. J. Bacteriol., 172, 4315-4321) disrupted in the transcriptional unit of the known *Bacillus subtilis* cellulase gene, resulting in cellulase negative cells. The disruption was performed essentially as described in (Eds. A.L. Sonenshein, J.A. Hoch and Richard Losick (1993) *Bacillus subtilis* and other Gram-Positive Bacteria, American Society for microbiology, p.618).

Competent cells were prepared and transformed as described by Yasbin, R.E., Wilson, G.A. and Young, F.E. (1975) Transformation and transfection in lysogenic strains of *Bacillus subtilis*: evidence for selective induction of prophage in competent cells. J. Bacteriol, 121:296-304.

Plasmids

pSJ1678 (as decribed in detail in WO 94/19454 which is hereby incorporated by reference in its entirety).

pMOL944:

This plasmid is a pUB110 derivative essentially containing elements making the plasmid propagatable in *Bacillus subtilis*, kanamycin resistance gene and having a strong promoter and signal peptide cloned from the amyL gene of *B.licheniformis* ATCC14580. The signal peptide contains a SacII site making it convenient to clone the DNA encoding the mature part of a protein in-fusion with the signal peptide. This results in the expression of a Pre-protein which is directed towards the exterior of the cell.

The plasmid was constructed by means of conventional genetic engineering techniques which are briefly described in the following.

Construction of pMOL944:

The pUB110 plasmid (McKenzie, T. et al., 1986, Plasmid 15:93-103) was digested with the unique restriction enzyme NciI. A PCR fragment amplified from the amyL promoter encoded on the plasmid pDN1981 (P.L. Jørgensen et al.,1990, Gene, 96, p37-41.) was digested with NciI and inserted in the NciI digested pUB110 to give the plasmid pSJ2624.

The two PCR primers used have the following sequences:

LWN5494 5′-GTCGCCGGGGCGGCCGCTATCAATTGGTAACTGTATCTCAGC -3′

LWN5495 5′-GTCGCCCGGGAGCTCTGATCAGGTACCAAGCTTGTCGACCTGCAGAA TGAGGCAGCAAGAAGAT -3′

The primer #LWN5494 inserts a NotI site in the plasmid.

The plasmid pSJ2624 was then digested with SacI and NotI and a new PCR fragment amplified on amyL promoter encoded on the pDN1981 was digested with SacI and NotI and this DNA fragment was inserted in the SacI-NotI digested pSJ2624 to give the plasmid pSJ2670.

This cloning replaces the first amyL promoter cloning with the same promoter but in the opposite direction. The two primers used for PCR amplification have the following sequences:

#LWN5938 5`-GTCGGCGGCCGCTGATCACGTACCAAGCTTGTCGACCTGCAGAATG AGGCAGCAAGAAGAT -3′

#LWN5939 5`-GTCGGAGCTCTATCAATTGGTAACTGTATCTCAGC -3′

The plasmid pSJ2670 was digested with the restriction enzymes PstI and BclI and a PCR fragment amplified from a cloned

DNA sequence encoding the alkaline amylase SP722 (disclosed in the International Patent Application published as WO95/26397 which is hereby incorporated by reference in its entirety) was digested with PstI and BclI and inserted to give the plasmid pMOL944. The two primers used for PCR amplification have the following sequence:

#LWN7864 5` -AACAGCTGATCACGACTGATCTTTTAGCTTGGCAC-3´

#LWN7901 5` -AACTGCAGCCGCGGCACATCATAATGGGACAAATGGG -3´

The primer #LWN7901 inserts a SacII site in the plasmid.

Cloning of the mannanase gene from *Bacillus agaradherens*

Genomic DNA preparation:

Strain *Bacillus agaradherens* NCIMB 40482 was propagated in liquid medium as described in WO94/01532. After 16 hours incubation at 30°C and 300 rpm, the cells were harvested, and genomic DNA isolated by the method described by Pitcher et al. (Pitcher, D. G., Saunders, N. A., Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol., 8, 151-156).

Genomic library construction:

Genomic DNA was partially digested with restriction enzyme Sau3A, and size-fractionated by electrophoresis on a 0.7 % agarose gel. Fragments between 2 and 7 kb in size was isolated by electrophoresis onto DEAE-cellulose paper (Dretzen, G., Bellard, M., Sassone-Corsi, P., Chambon, P. (1981) A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem., 112, 295-298).

Isolated DNA fragments were ligated to BamHI digested pSJ1678 plasmid DNA, and the ligation mixture was used to trans-

form *E. coli* SJ2.

Identification of positive clones:

A DNA library in *E. coli*, constructed as described above, was screened on LB agar plates containing 0.2% AZCL-galactomannan (Megazyme) and 9 µg/ml Chloramphenicol and incubated overnight at 37°C. Clones expressing mannanase activity appeared with blue diffusion halos. Plasmid DNA from one of these clone was isolated by Qiagen plasmid spin preps on 1 ml of overnight culture broth (cells incubated at 37°C in TY with 9 µg/ml Chloramphenicol and shaking at 250 rpm).

This clone (MB525) was further characterized by DNA sequencing of the cloned Sau3A DNA fragment. DNA sequencing was carried out by primerwalking, using the Taq deoxy-terminal cycle sequencing kit (Perkin-Elmer, USA), fluorescent labelled terminators and appropriate oligonucleotides as primers.

Analysis of the sequence data was performed according to Devereux *et al.* (1984) Nucleic Acids Res. 12, 387-395. The sequence encoding the mannanase is shown in SEQ ID No 1. The derived protein sequence is shown in SEQ ID No.2.

Subcloning and expression of mannanase in *B.subtilis*:

The mannanase encoding DNA sequence of the invention was PCR amplified using the PCR primer set consisting of these two oligo nucleotides:

Mannanase.upper.SacII

5'-CAT TCT GCA GCC GCG GCA GCA AGT ACA GGC TTT TAT GTT GAT GG-3'

Mannanase.lower.NotI

5'-GAC GAC GTA CAA GCG GCC GCG CTA TTT CCC TAA CAT GAT GAT ATT TTC G -3'

Restriction sites SacII and NotII are underlined. Chromosomal DNA isolated from *B.agaradherens* NCIMB 40482 as described above was used as template in a PCR reaction using Amplitaq DNA Polymerase (Perkin Elmer) according to manufacturers instructions. The PCR reaction was set up in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % (w/v) gelatin) containing 200 µM of each dNTP, 2.5 units of AmpliTaq polymerase (Perkin-Elmer, Cetus, USA) and 100 pmol of each primer.

The PCR reaction was performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 1 min followed by thirty cycles of PCR performed using a cycle profile of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, and extension at 72°C for 2 min. Five-µl aliquots of the amplification product was analysed by electrophoresis in 0.7 % agarose gels (NuSieve, FMC). The appearance of a DNA fragment size 1.4 kb indicated proper amplification of the gene segment.

Subcloning of PCR fragment.

Fortyfive-µl aliquots of the PCR products generated as described above were purified using QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. 5 µg of pMOL944 and twentyfive-µl of the purified PCR fragment was digested with SacII and NotI, electrophoresed in 0.8% low gelling temperature agarose (SeaPlaque GTG, FMC) gels, the relevant fragments were excised from the gels, and purified using QIAquick Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated PCR DNA fragment was then ligated to the SacII-NotI digested and purified pMOL944. The ligation was performed overnight at 16°C using 0.5µg of each DNA fragment, 1 U of T4 DNA ligase and T4 ligase buffer

(Boehringer Mannheim, Germany).

The ligation mixture was used to transform competent *B.subtilis* PL2306. The transformed cells were plated onto LBPG-10 µg/ml of Kanamycin plates. After 18 hours incubation at 37°C colonies were seen on plates. Several clones were analysed by isolating plasmid DNA from overnight culture broth.

One such positive clone was restreaked several times on agar plates as used above, this clone was called MB594. The clone MB594 was grown overnight in TY-10 µg/ml kanamycin at 37°C, and next day 1 ml of cells were used to isolate plasmid from the cells using the Qiaprep Spin Plasmid Miniprep Kit #27106 according to the manufacturers recommendations for *B.subtilis* plasmid preparations. This DNA was DNA sequenced and revealed the DNA sequence corresponding to the mature part of the mannanase, i.e. positions 94-1404 of the appended SEQ ID NO:3. The derived mature protein is shown in SEQ ID NO:4. It will appear that the 3′ end of the mannanse encoded by the sequence of SEQ ID NO:1 was changed to the one shown in SEQ ID NO:3 due to the design of the lower primer used in the PCR. The resulting amino acid sequence is shown in SEQ ID NO:4 and it is apparent that the C terminus of the SEQ ID NO:2 (SHHVREIGVQFSAADNSSGQTALYVDNVTLR) is changed to the C terminus of SEQ ID NO:4 (IIMLGK).

Media:

TY (as described in Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995).

LB agar (as described in Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995).

LBPG is LB agar (see above) supplemented with 0.5% Glucose and 0.05 M potassium phosphate, pH 7.0

BPX media is described in EP 0 506 780 (WO 91/09129).

The following examples illustrate the invention.

EXAMPLE 1

Expression, purification and characterisation of mannanase from *Bacillus agaradherens*

The clone MB 594 obtained as described above under Materials and Methods was grown in 25 x 200ml BPX media with 10 µg/ml of Kanamycin in 500ml two baffled shakeflasks for 5 days at 37°C at 300 rpm.

6500 ml of the shake flask culture fluid of the clone MB 594 (batch #9813) was collected and pH adjusted to 5.5. 146 ml of cationic agent (C521) and 292 ml of anionic agent (A130) was added during agitation for flocculation. The flocculated material was separated by centrifugation using a Sorval RC 3B centrifuge at 9000 rpm for 20 min at 6°C. The supernatant was clarified using Whatman glass filters GF/D and C and finally concentrated on a filtron with a cut off of 10 kDa.

750 ml of this concentrate was adjusted to pH 7.5 using sodium hydroxide. The clear solution was applied to anion-exchange chromatography using a 900 ml Q-Sepharose column equilibrated with 50 mmol Tris pH 7.5. The mannanase activity bound was eluted using a sodium chloride gradient.

The pure enzyme gave a single band in SDS-PAGE with a molecular weight of 38 kDa.

The amino acid sequence of the mannanase enzyme, i.e. the translated DNA sequence, is shown in SEQ ID No.2.

Determination of kinetic constants:

Substrate: Locust bean gum (carob) and reducing sugar analysis (PHBAH). Locust bean gum from Sigma (G-0753).

Kinetic determination using different concentrations of locust bean gum and incubation for 20 min at 40°C at pH 10 gave

Kcat: 467 per sec.

K_m: 0.08 gram per l

MW: 38kDa

pI (isoelectric point): 4.2

The temperature optimum of the mannanase was found to be 60°C.

The pH activity profile showed maximum activity between pH 8 and 10.

DSC differential scanning calometry gives 77°C as melting point at pH 7.5 in Tris buffer indicating that this enzyme is very termostable.

Detergent compatibility using 0.2% AZCL-Galactomannan from carob as substrate and incubation as described above at 40°C shows excellent compability with conventional liquid detergents and good compability with conventional powder detergents.

EXAMPLE 2

Use of the enzyme of the invention in detergents

The purified enzyme obtained as described in example 1 (batch #9813) showed improved cleaning performance when tested at a level of 1 ppm in a miniwash test using a conventional commercial liquid detergent. The test was carried out under conventional North American wash conditions.

LITERATURE

Lever, M. (1972) A new reaction for colormetric determination of carbohydrates. Anal. Biochem. *47*, 273-279.

N. C. Carpita and D. M. Gibeaut (1993) The Plant Journal **3**:1-30.

Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B. R., Sjøholm, C. (1990) Cloning of aldB, which encodes alpha-acetolactate decarboxylase, an exoenzyme from *Bacillus brevis*. J. Bacteriol. **172**:4315-4321.

SEQUENCE LISTING

Organism: *Bacillus agaradherens*, NCIMB 40482.

Enzyme: Mannanase

SEQ ID NO:1

SEQUENCE CHARACTERISITICS:
LENGTH: 1407 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE

FEATURE:
NAME/KEY: CDS
LOCATION: 1-1482

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

ATGAAAAAAAAGTTATCACAGATTTATCATTTAATTATTTGCACACTTATAATAAGTGTGGGAA
TAATGGGGATTACAACGTCCCCATCAGCAGCAAGTACAGGCTTTTATGTTGATGGCAATACGTT
ATATGACGCAAATGGGCAGCCATTTGTCATGAGAGGTATTAACCATGGACATGCTTGGTATAAA
GACACCGCTTCAACAGCTATTCCTGCCATTGCAGAGCAAGGCGCCAACACGATTCGTATTGTTT
TATCAGATGGCGGTCAATGGGAAAAAGACGACATTGACACCATTCGTGAAGTCATTGAGCTTGC
GGAGCAAAATAAAATGGTGGCTGTCGTTGAAGTTCATGATGCCACGGGTCGCGATTCGCGCAGT
GATTTAAATCGAGCCGTTGATTATTGGATAGAAATGAAAGATGCGCTTATCGGTAAAGAAGATA
CGGTTATTATTAACATTGCAAACGAGTGGTATGGGAGTTGGGATGGCTCAGCTTGGGCCGATGG
CTATATTGATGTCATTCCGAAGCTTCGCGATGCCGGCTTAACACACACCTTAATGGTTGATGCA
GCAGGATGGGGGCAATATCCGCAATCTATTCATGATTACGGACAAGATGTGTTTAATGCAGATC
CGTTAAAAAATACGATGTTCTCCATCCATATGTATGAGTATGCTGGTGGTGATGCTAACACTGT
TAGATCAAATATTGATAGAGTCATAGATCAAGACCTTGCTCTCGTAATAGGTGAATTCGGTCAT
AGACATACTGATGGTGATGTTGATGAAGATACAATCCTTAGTTATTCTGAAGAAACTGGCACAG
GGTGGCTCGCTTGGTCTTGGAAAGGCAACAGTACCGAATGGGACTATTTAGACCTTTCAGAAGA
CTGGGCTGGTCAACATTTAACTGATTGGGGGAATAGAATTGTCCACGGGGCCGATGGCTTACAG
GAAACCTCCAAACCATCCACCGTATTTACAGATGATAACGGTGGTCACCCTGAACCGCCAACTG
CTACTACCTTGTATGACTTTGAAGGAAGCACACAAGGGTGGCATGGAAGCAACGTGACCGGTGG
CCCTTGGTCCGTAACAGAATGGGGTGCTTCAGGTAACTACTCTTTAAAAGCCGATGTAAATTTA
ACCTCAAATTCTTCACATGAACTGTATAGTGAACAAAGTCGTAATCTACACGGATACTCTCAGC
TCAACGCAACCGTTCGCCATGCCAATTGGGGAAATCCCGGTAATGGCATGAATGCAAGACTTTA
CGTGAAAACGGGCTCTGATTATACATGGCATAGCGGTCCTTTTACACGTATCAATAGCTCCAAC
TCAGGAACAACGTTATCTTTTGATTTAAACAACATCGAAAATAGTCATCATGTTAGGGAAATAG
GCGTGCAATTTTCAGCGGCAGATAATAGCAGTGGTCAAACTGCTCTATACGTTGATAACGTTAC

TTTAAGATAG

SEQ ID NO:2

SEQUENCE CHARACTERISITICS:
LENGTH: 493 amino acids
TYPE: amino acid
TOPOLOGY: linear

MOLECULE TYPE: protein
SEQUENCE DESCRIPTION: SEQ ID NO: 2

MKKKLSQIYHLIICTLIISVGIMGITTSPSAASTGFYVDGNTLYDANGQPFVMRGINHGHAWYK
DTASTAIPAIAEQGANTIRIVLSDGGQWEKDDIDTIREVIELAEQNKMVAVVEVHDATGRDSRS
DLNRAVDYWIEMKDALIGKEDTVIINIANEWYGSWDGSAWADGYIDVIPKLRDAGLTHTLMVDA
AGWGQYPQSIHDYGQDVFNADPLKNTMFSIHMYEYAGGDANTVRSNIDRVIDQDLALVIGEFGH
RHTDGDVDEDTILSYSEETGTGWLAWSWKGNSTEWDYLDLSEDWAGQHLTDWGNRIVHGADGLQ
ETSKPSTVFTDDNGGHPEPPTATTLYDFEGSTQGWHGSNVTGGPWSVTEWGASGNYSLKADVNL
TSNSSHELYSEQSRNLHGYSQLNATVRHANWGNPGNGMNARLYVKTGSDYTWHSGPFTRINSSN
SGTTLSFDLNNIENSHHVREIGVQFSAADNSSGQTALYVDNVTLR

SEQ ID NO:3

DNA, 1407 base pairs

ATGAAAAAAAAGTTATCACAGATTTATCATTTAATTATTTGCACACTTATAATAAGTGTGGGAA
TAATGGGGATTACAACGTCCCCATCAGCAGCAAGTACAGGCTTTTATGTTGATGGCAATACGTT
ATATGACGCAAATGGGCAGCCATTTGTCATGAGAGGTATTAACCATGGACATGCTTGGTATAAA
GACACCGCTTCAACAGCTATTCCTGCCATTGCAGAGCAAGGCGCCAACACGATTCGTATTGTTT
TATCAGATGGCGGTCAATGGGAAAAAGACGACATTGACACCATTCGTGAAGTCATTGAGCTTGC
GGAGCAAAATAAAATGGTGGCTGTCGTTGAAGTTCATGATGCCACGGGTCGCGATTCGCGCAGT
GATTTAAATCGAGCCGTTGATTATTGGATAGAAATGAAAGATGCGCTTATCGGTAAAGAAGATA
CGGTTATTATTAACATTGCAAACGAGTGGTATGGGAGTTGGGATGGCTCAGCTTGGGCCGATGG
CTATATTGATGTCATTCCGAAGCTTCGCGATGCCGGCTTAACACACACCTTAATGGTTGATGCA
GCAGGATGGGGGCAATATCCGCAATCTATTCATGATTACGGACAAGATGTGTTTAATGCAGATC
CGTTAAAAAATACGATGTTCTCCATCCATATGTATGAGTATGCTGGTGGTGATGCTAACACTGT
TAGATCAAATATTGATAGAGTCATAGATCAAGACCTTGCTCTCGTAATAGGTGAATTCGGTCAT
AGACATACTGATGGTGATGTTGATGAAGATACAATCCTTAGTTATTCTGAAGAAACTGGCACAG
GGTGGCTCGCTTGGTCTTGGAAAGGCAACAGTACCGAATGGGACTATTTAGACCTTTCAGAAGA
CTGGGCTGGTCAACATTTAACTGATTGGGGGAATAGAATTGTCCACGGGGCCGATGGCTTACAG
GAAACCTCCAAACCATCCACCGTATTTACAGATGATAACGGTGGTCACCCTGAACCGCCAACTG
CTACTACCTTGTATGACTTTGAAGGAAGCACACAAGGGTGGCATGGAAGCAACGTGACCGGTGG
CCCTTGGTCCGTAACAGAATGGGGTGCTTCAGGTAACTACTCTTTAAAAGCCGATGTAAATTTA
ACCTCAAATTCTTCACATGAACTGTATAGTGAACAAAGTCGTAATCTACACGGATACTCTCAGC

TCAACGCAACCGTTCGCCATGCCAATTGGGGAAATCCCGGTAATGGCATGAATGCAAGACTTTA
CGTGAAAACGGGCTCTGATTATACATGGCATAGCGGTCCTTTTACACGTATCAATAGCTCCAAC
TCAGGAACAACGTTATCTTTTGATTTAAACAACATCGAAAATATCATCATGTTAGGGAAATAG

SEQ ID NO:4

Amino acid, 468 residues

MKKKLSQIYHLIICTLIISVGIMGITTSPSAASTGFYVDGNTLYDANGQPFVMRGINHGHAWYK
DTASTAIPAIAEQGANTIRIVLSDGGQWEKDDIDTIREVIELAEQNKMVAVVEVHDATGRDSRS
DLNRAVDYWIEMKDALIGKEDTVIINIANEWYGSWDGSAWADGYIDVIPKLRDAGLTHTLMVDA
AGWGQYPQSIHDYGQDVFNADPLKNTMFSIHMYEYAGGDANTVRSNIDRVIDQDLALVIGEFGH
RHTDGDVDEDTILSYSEETGTGWLAWSWKGNSTEWDYLDLSEDWAGQHLTDWGNRIVHGADGLQ
ETSKPSTVFTDDNGGHPEPPTATTLYDFEGSTQGWHGSNVTGGPWSVTEWGASGNYSLKADVNL
TSNSSHELYSEQSRNLHGYSQLNATVRHANWGNPGNGMNARLYVKTGSDYTWHSGPFTRINSSN
SGTTLSFDLNNIENIIMLGK

CLAIMS

1. A mannanase which is
(a) a polypeptide produced by *Bacillus agaradherens*, NCIMB 40482, or
(b) a polypeptide comprising an amino acid sequence as shown in positions 32-343 of SEQ ID NO:2, or
(c) an analogue of the polypeptide defined in (a) or (b) which is at least 70% homologous with said polypeptide, or is derived from said polypeptide by substitution, deletion or addition of one or several amino acids, or is immunologically reactive with a polyclonal antibody raised against said polypeptide in purified form.

2. An isolated polynucleotide molecule encoding a polypeptide having mannanase activity selected from the group consisting of:
(a) polynucleotide molecules comprising a nucleotide sequence as shown in SEQ ID NO:1 from nucleotide 97 to nucleotide 1029;
(b) polynucleotide molecules that encode a polypeptide that is at least 70% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 32 to amino acid residue 343; and
(c) degenerate nucleotide sequences of (a) or (b).

3. The isolated polynucleotide molecule according to claim 2, wherein the polynucleotide is DNA.

4. An isolated polynucleotide molecule encoding a polypeptide having mannanase activity which polynucleotide molecule hybridizes to a denatured double-stranded DNA probe under medium stringency conditions, wherein the probe is selected from the group consisting of DNA probes comprising the sequence shown in positions 97-1029 of SEQ ID NO:1 and DNA probes comprising a

subsequence of positions 97-1029 of SEQ ID NO:1 having a length of at least about 100 base pairs.

5. An expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment selected from the group consisting of (a) polynucleotide molecules encoding a polypeptide having mannanase activity comprising a nucleotide sequence as shown in SEQ ID NO:1 from nucleotide 97 to nucleotide 1029, (b) polynucleotide molecules encoding a polypeptide having mannanase activity that is at least 70% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 32 to amino acid residue 343, and (c) degenerate nucleotide sequences of (a) or (b); and a transcription terminator.

6. A cultured cell into which has been introduced an expression vector according to claim 5, wherein said cell expresses the polypeptide encoded by the DNA segment.

7. An isolated polypeptide having mannanase activity selected from the group consisting of:
(a) polypeptide molecules comprising an amino acid sequence as shown in SEQ ID NO: 2 from residue 32 to residue 343; and
(b) polypeptide molecules that are at least 70% identical to the amino acids of SEQ ID NO: 2 from amino acid residue 32 to amino acid residue 343.

8. The polypeptide according to claim 7 which is produced by *Bacillus agaradherens*.

9. An enzyme preparation comprising a purified polypeptide according to claim 7.

10. A method of producing a polypeptide having mannanase activity comprising culturing a cell into which has been introduced an expression vector according to claim 5, whereby said cell expresses a polypeptide encoded by the DNA segment; and recovering the polypeptide.

11. The preparation according to claim 9 which further comprises one or more enzymes selected from the group consisting of proteases, cellulases (endoglucanases), β-glucanases, hemicellulases, lipases, peroxidases, laccases, α-amylases, glucoamylases, cutinases, pectinases, reductases, oxidases, phenoloxidases, ligninases, pullulanases, pectate lyases, xyloglucanases, xylanases, pectin acetyl esterases, polygalacturonases, rhamnogalacturonases, pectin lyases, other mannanases, pectin methylesterases, cellobiohydrolases, transglutaminases; or mixtures thereof.

12. An isolated enzyme having mannanase activity, in which the enzyme is (i) free from homologous impurities, and (ii) produced by the method according to claim 9.

13. A method for improving the properties of cellulosic or synthetic fibres, yarn, woven or non-woven fabric in which method the fibres, yarn or fabric is treated with an effective amount of the preparation according to claim 9 or an effective amount of the enzyme according to claim 1.

14. The method according to claim 13, wherein the enzyme preparation or the enzyme is used in a desizing process step.

15. A method for degradation or modification of plant material in which method the plant material is treated with an effective amount of the preparation according to claim 9 or an effective amount of the enzyme according to claim 1.

16. The method according to claim 14 wherein the plant material is recycled waste paper; mechanical, chemical, semichemical, kraft or other paper-making pulps; fibres subjected to a retting process; or guar gum or locust bean gum containing material.

17. A method for processing liquid coffee extract, in which method the coffee extract is treated with an effective amount of the preparation according to claim 9 or an effective amount of the enzyme according to claim 1.

18. A cleaning composition comprising the enzyme preparation according to claim 9 or the enzyme according to claim 1.

19. The cleaning composition according to claim 18 which further comprises a bioscouring enzyme selected from cellulases, amylases, pectin degrading enzymes and/or xyloglucanases; and another detergent ingredient.

20. The cleaning composition according to claim 18 wherein said enzyme or enzyme preparation is present at a level of from 0.0001% to 2%, preferably from 0.0005% to 0.5%, more preferably from 0.001% to 0.1% pure enzyme by weight of total composition.

21. The cleaning composition according to claim 19 wherein the bioscouring enzyme is present at a level of from 0.0001% to 2%, preferably from 0.0005% to 0.5%, more preferably from 0.001% to 0.1% pure enzyme by weight of total composition.

22. The cleaning composition according to claim 19 wherein the biouscouring enzyme is an amylase.

23. The cleaning composition according to claim 22 which further comprises another bioscouring enzyme selected from cellulase, pectin degrading enzyme and/or xyloglucanase.

24. The cleaning composition according to claim 19 wherein the bioscouring enzyme is alkaline.

25. The cleaning composition according to claim 19 which further comprises a surfactant selected from anionic, nonionic, cationic surfactant, and/or mixtures thereof.

26. The cleaning composition according to claim 19 which further comprises a bleaching agent.

27. The cleaning composition according to claim 19 which further comprises a builder.

28. A fabric softening composition according to claim 19 which further comprises a cationic surfactant comprising two long chain lengths.

29. A process for machine treatment of fabrics which process comprises treating fabric during a washing cycle of a machine washing process with a washing solution containing the enzyme preparation according to claim 9 or the enzyme according to claim 1.

30. Use of the enzyme preparation according to claim 9 or the enzyme according to claim 1 together with a bioscouring enzyme selected from cellulase, amylase, pectin degrading enzyme and/or xyloglucanase in a cleaning composition for fabric cleaning and/or fabric stain removal.

31. Use of the enzyme preparation according to claim 9 or the enzyme according to claim 1 together with a bioscouring enzyme selected from cellulase, amylase, pectin degrading enzyme and/or xyloglucanase in a cleaning composition for cleaning hard surfaces such as floors, walls, bathroom tile and the like.

32. Use of the enzyme preparation according to claim 9 or the enzyme according to claim 1 together with a bioscouring enzyme selected from cellulase, amylase, pectin degrading enzyme and/or xyloglucanase in a cleaning composition for hand and machine dishwashing.

33. Use of the enzyme preparation according to claim 9 or the enzyme according to claim 1 together with a bioscouring enzyme selected from cellulase, amylase, pectin degrading enzyme and/or xyloglucanase in a cleaning composition for oral, dental, contact lenses and personal cleaning applications.

ABSTRACT

NOVEL MANNANASES

Novel mannanases comprising an amino acid sequence as shown in positions 32-343 of SEQ ID NO:2 or their homologues may be derived from eg *Bacillus agaradherens*, NCIMB 40482, or may be encoded by polynucleotide molecules comprising a nucleotide sequence as shown in SEQ ID NO: 1 from nucleotide 97 to nucleotide 1029, polynucleotide molecules that encode a polypeptide that is at least 70% identical to the amino acid sequence of SEQ ID NO: 2 from amino acid residue 32 to amino acid residue 343, or degenerate nucleotide sequences thereof. The mannanases are alkaline and are useful e.g. in cleaning compositions, in a fracturing fluid useful to fracture a subterranean formation, for modifying plant material, and for treatment of cellulosic fibres.